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(57) Abstract

Methods for increasing the level of free, biologically active proteins, including insulin-like growth factors, and for treating IGF-responsive conditions are provided. The methods generally comprise administering one or more ligand inhibitors that inhibit the binding of the protein to one or more insulin-like growth factor binding proteins. Suitable ligand inhibitors include analogs of IGF-I or IGF-II and small molecule inhibitors.

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Description

LIGAND INHIBITORS OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND METHODS OF USE THEREFOR

Technical Field

The present invention relates generally to compositions and methods for the treatment of conditions responsive to the administration of insulin, human growth hormone, insulin-like growth factor, and/or other proteins that bind to insulin-like growth factor binding proteins. The invention is more particularly related to ligand inhibitors that inhibit the binding of insulin-like growth factor to insulin-like growth factor binding proteins, and to the use of such inhibitors for administration (*e.g.*, orally) to patients for the treatment of diabetes, neurodegenerative diseases and other disorders.

Background of the Invention

Insulin-like growth factors (IGFs) are polypeptide hormones that are structurally similar to each other and to insulin. Two IGFs, known as IGF-I and IGF-II, have been identified, and both have a variety of metabolic actions and affect the growth of multiple cell types (*see, e.g.*, LeRoith and Roberts, "Insulin-like Growth Factors," *Ann. NY Acad. Sci. 692*:1-9, 1993). IGF-I is a 70 amino acid peptide with a molecular weight of 7649 and three disulfide bridges. Its actions *in vivo* include the mediation of growth hormone actions and bone deposition and maturation. IGF-I also mimics the action of insulin, and the IGF-I receptor has high homology to the insulin receptor. IGF-II is strongly homologous to IGF-I and this factor plays a role in, for example, bone remodeling and brain cell maintenance and differentiation.

While IGF-I is present in a wide variety of body tissues, it is normally found in an inactive form in which it is bound to an IGF binding protein (IGFBP or BP). Six related BPs are known and have been designated IGFBP1-IGFBP6 (*see, e.g.*, Holly and Martin, "Insulin-like Growth Factor Binding Proteins: A Review of Methodological Aspects of Their Purification, Analysis and Regulation," *Growth*

Regul. 4(Suppl 1):20-30, 1994; Langford et al., "The Insulin-like Growth Factor-I/Binding Protein Axis: Physiology, Phytophysiology and Therapeutic Manipulation," *Eur. J. Clin. Invest. 23(9)*:503-16, 1993). BPs play an important role in IGF regulation by exerting inhibitory and/or stimulatory effects on IGF action. For example, about 90% of circulating IGF-I is present in a trimolecular complex containing IGFBP-3 and acid labile subunit (ALS). The IGF-I within such complexes is unable to bind to surface receptors, and is therefore biologically inactive. IGF-I present within the trimolecular complex also has a substantially longer half-life than uncomplexed IGF-I.

Attempts have been made to treat a wide variety of diseases by administration of IGF-I, IGF-II or an IGF binding protein. For example, the use of IGF-I for the treatment of cardiac disorders, intestinal disorders and osteoporosis are described in U.S. Patent No. 5,126,324, WO 91/12018 and European Patent Application 560,723, respectively, and the use of IGF-I for enhancing growth is described in U.S. Patent No. 5,126,324. IGF-I has also been studied for use in treating insulin-resistant states and diabetes (*see, e.g.*, Clemmons and Underwood, "Uses of Human Insulin-Like Growth Factor-I in Clinical Conditions," *J. Clin Endocrinol. Metab. 79(1)*:4-6, 1994; Langford et al., "The Insulin-like Growth Factor-I/Binding Protein Axis: Physiology, Phytophysiology and Therapeutic Manipulation," *Eur. J. Clin. Invest. 23(9)*:503-16, 1993). Therapies involving the administration of antibodies raised against IGF-I and specific binding proteins are described, for example, in WO 94/04569 and WO 92/14834, respectively.

Like treatment with insulin or growth hormone, however, treatment with intravenous IGF or antibodies thereto generally requires repeated intravenous injection, resulting in a high cost and practical difficulties for patients. Such treatments can also induce side effects due, for example, to the inability to target specific tissues within the body. Further, the scope of treatment is limited to the tissues that may be reached by intravenous hormone administration.

Accordingly, there is a need in the art for improved efficiency and control in treating conditions responsive to IGF and/or other proteins that bind to insulin-like growth factor binding proteins. The present invention fulfills these needs and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention provides therapeutic and screening methods employing ligand inhibitors that inhibit the binding of proteins such as insulin-like growth factors to insulin-like growth factor binding proteins. In one aspect, the present invention provides a method for increasing the level of free, biologically active insulin-like growth factor in a patient, comprising administering to a patient one or more ligand inhibitors that inhibit the binding of an insulin-like growth factor to one or more insulin-like growth factor binding proteins, and thereby increasing the level of free, biologically active insulin-like growth factors within the patient.

In a related aspect, methods are provided for treating an IGF-responsive condition in a patient, comprising administering to a patient one or more ligand inhibitors that inhibit the binding of an insulin-like growth factor to one or more insulin-like growth factor binding proteins, and thereby alleviating an IGF-responsive condition in a patient.

In another aspect, the present invention provides a pharmaceutical composition comprising: (a) one or more ligand inhibitors that inhibit the binding of an insulin-like growth factor to one or more insulin-like growth factor binding proteins; and (b) a physiologically acceptable carrier.

In yet another aspect, the present invention provides a method for screening for a small molecule inhibitor that inhibits binding of an insulin-like growth factor to an insulin-like growth factor binding protein, comprising: (a) combining an insulin-like growth factor with an insulin-like growth factor binding protein in a solution containing a candidate small molecule, such that the binding protein and the growth factor are capable of forming a complex; and (b) determining the amount of complex in the solution, relative to a predetermined level of binding in the absence of the small molecule, and therefrom evaluating the ability of the small molecule to inhibit binding of an insulin-like growth factor to an insulin-like growth factor binding protein.

In still another aspect, methods are provided for increasing the level of a free protein in a patient, comprising administering to a patient one or more ligand

inhibitors that inhibit the binding of a protein to one or more insulin-like growth factor binding proteins, and thereby increasing the level of the free protein within the patient.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

Brief Description of the Drawings

Figure 1 is a graph that depicts the relative amount of binding, expressed as cpm, of ^{125}I-labeled IGF-I tracer to IGFBP-3, in the presence of varying amounts of [T59]hIGF-I, hIGF-I and [L24,59,60,A31]hIGF-I.

Figure 2 is a graph that presents the relative amount of DNA synthesis, expressed as cpm of incorporated [^{3}H]-thymidine, of 3T3 cells in the presence of varying amounts of [T59]hIGF-I and [L24,59,60,A31]hIGF-I.

Figure 3 is a graph that depicts the relative amount of DNA synthesis, expressed as cpm of incorporated [^{3}H]-thymidine, of 3T3 cells in the presence of medium only (column 1), 10 nM [T59]hIGF-I (column 2) and 10 nM [T59]hIGF-I plus 25 nM IGFBP-3 (column 3). The figure also shows the relative amount of DNA synthesis in the presence of 10 nM [T59]hIGF-I plus 25 nM IGFBP-3, with the addition of varying amounts of [L24,59,60,A31]hIGF-I.

Figure 4 is a graph that shows the decrease in blood glucose levels (in mg/dl) over time (expressed in minutes) following administration of [L24,59,60,A31]hIGF-I to diabetic NOD mice.

Figure 5 is a pair of graphs that depict the effect of insulin and IGFBP3-LI ([L24,59,60,A31]hIGF-I) on plasma glucose in rats treated systemically with glucose.

Detailed Description of the Invention

As noted above, the present invention is generally directed to methods employing ligand inhibitors for increasing the level of a free protein, such as biologically active IGF, in one or more tissues within a patient. The proteins affected

by ligand inhibitors as described herein are generally proteins that bind to one or more IGF binding proteins. Increasing the level of such a protein within a patient may generally be useful in the treatment of a variety of conditions. Within the context of the present invention, "IGF" refers to one or more insulin-like growth factors (*i.e.*, IGF-I and/or IGF-II). IGFs are peptide hormones, and the sequences of IGF-I and IGF-II in humans and other species have been determined. Human IGF-I (hIGF-I) is a 70 amino acid peptide which has the sequence shown in Figure 1 (SEQ ID NO:1). "Free" protein, such as IGF, refers to protein that is not complexed or bound to an IGF binding protein.

An "IGF binding protein" (IGFBP or BP) is any protein that binds to IGF-I and/or IGF-II *in vivo*, resulting in the inhibition of IGF binding to one or more cell surface receptors, or soluble forms thereof. A BP binds to IGF (*i.e.*, forms a complex) through noncovalent interactions. IGF binding proteins contemplated within the context of the present invention include IGFBP-1, -2, -3, -4, -5 and -6. In particular, IGFBP-3, the most abundant binding protein in adult serum, has a high affinity for both IGF-I and IGF-II. Binding to IGFBP-3 increases the half life of IGF-I from about 10 minutes to approximately 15 hours (*see* Langford and Miell, *Eur. J. Clin. Invest. 23*:503-526, 1993), and is therefore important for controlling the level of IGF-I in the circulation.

A "ligand inhibitor," within the context of the present invention, is any molecule (other than an antibody to IGF-I or IGF-II) that is capable of inhibiting the binding of one or more proteins, especially IGF-I and/or IGF-II, to one or more IGFBPs. Due to the similarity between the structures of IGF-I and IGF-II, it will be apparent that many ligand inhibitors will inhibit the binding of both IGF-I and IGF-II to an IGFBP. In some cases, the binding of one IGF will be inhibited to a greater extent than that of the other IGF. In other cases, however, a ligand inhibitor may be specific for IGF-I or IGF-II. A ligand inhibitor may displace IGF from a complex with a BP, thereby causing bound IGF to become free IGF. Such displacement may be reversible or irreversible. A ligand inhibitor may also block the binding of free IGF to a BP because of a high affinity for either IGF or one or more BPs. For example, a ligand inhibitor may bind to IGF within a BP binding site, or may bind to a BP at an IGF

binding site. Alternatively, a ligand inhibitor may bind to IGF or a BP at a site that is not such a binding site, and inhibit complex formation through an allosteric interaction. A ligand inhibitor having a lower affinity for IGF or one or more BPs may also inhibit the binding of free IGF to a BP when present at high enough concentrations. Similar mechanisms of inhibition may also be observed for ligand inhibitors directed against other proteins that bind to a BP.

As used herein, a molecule "inhibits" binding of a protein to an IGFBP if the level of free protein increases by about 10-30% or more. An increase in the level of free protein may generally be detected using a variety of assays known to those of ordinary skill in the art, such as imaging, radioimmunoassays and precipitation techniques as described herein. One such assay is described in Example 1, below. Preferably, the characteristics of the ligand inhibitor are such that it has a 100 fold selectivity to the IGFBP ($K_i \leq 10$ nM).

Ligand inhibitors include, but are not limited to, analogs of IGF-I or IGF-II and small molecule inhibitors. An IGF "analog" is a peptide that has an amino acid sequence that is substantially identical to a native IGF sequence, but that has one or more amino acid substitutions and/or modifications. Such substitutions and/or modifications may reduce the biological activity of the peptide (*i.e.*, decrease the affinity of the peptide for one or more cell surface receptors) without decreasing the ability of the peptide to bind to one or more BPs. A "small molecule inhibitor" is a ligand inhibitor that is a natural or synthesized non-peptide, organic molecule. Small molecule inhibitors are typically identified by screening libraries obtained from soil samples, plant extracts, marine microorganisms, fermentation broth, fungal broth, pharmaceutical chemical libraries, combinatorial libraries (both chemical and biological) and the like. Such libraries may be obtained from a variety of sources, both commercial and proprietary.

One preferred ligand inhibitor of the present invention is the [L24,A31,L59,L60] analog of hIGF-I. This analog (which has the sequence recited in SEQ ID NO:2) binds to IGFBP-3, but not to the IGF-I cell surface receptor. [L24,A31,L59,L60]hIGF-I may generally be prepared by techniques well known to those of ordinary skill in the art, such as by chemical synthesis.

It will be apparent to those of ordinary skill in the art that modifications may be made to the sequence of [L24,A31,L59,L60]hIGF-I such that the ability of the analog to inhibit the binding of IGF-I to IGFBP-3 is retained. Such variants are within the scope of the present invention, and may generally be identified by modifying the sequence and evaluating the inhibitory properties of the analog as described below. Preferably, a variant contains conservative substitutions, (*i.e.*, one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged). Amino acids suitable for conservative substitutions include those having functionally similar side chains. For example, a hydrophobic residue (*e.g.*, glycine, alanine, valine, leucine, isoleucine and methionine) may replace another such residue. Similarly, conservative substitutions may involve interchanging hydrophilic residues (*e.g.*, arginine and lysine, glutamine and asparagine, threonine and serine), basic residues (*e.g.*, lysine, arginine and histidine), and/or acidic residues (*e.g.*, aspartic acid and glutamic acid). Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids, or the chemical modification of amino acids, that have minimal influence on the inhibitory properties of the analog.

Small molecule inhibitors according to the present invention may be prepared using methods well known to those of ordinary skill in the art. According to a preferred method, a chemical library of small molecules as described above may be screened using a binding assay designed to detect molecules that displace a protein such as IGF from a binding protein. For example, a complex of radiolabeled IGF-I and a binding protein (such as BP-3) may be incubated in the presence of a candidate small molecule. Complexes (*i.e.*, noncovalent associations of IGF and binding protein) may then be separated from the remainder of the solution using, for example, polyethylene glycol precipitation. Those small molecules that bind to the complex and displace the growth factor from the binding protein may then be detected by a decrease in the amount of radiolabel precipitated. Those of ordinary skill in the art will recognize that a variety of other assay formats may be employed in such a screen, including two-site sandwich ELISAs, chemiluminescent assays and fluorescent assays.

IGF-I, IGF-II and binding proteins for use in such assays may generally be prepared by techniques known to those of ordinary skill in the art, such as those provided in Rechler, *Vitamins and Hormones 47*:1-114, 1993, and references cited therein. Appropriate techniques include chemical synthesis (described, for example, in Shimasaki et al., *J. Biol. Chem. 266*:10646-10653, 1991), purification from an appropriate biological sample (described, for example, in Shimonaka et al., *Biochem. Biophys. Res. Comm. 165*:189-195, 1989) and expression in a suitable host, such as yeast(described, for example, in Bayne et al., *J. Biol. Chem. 265*:15648-15652, 1990). In this regard, the IGF and binding proteins employed may be the native proteins, or may contain modifications, such as the addition of label or the addition or deletion of sequences that have minimal effect on the binding properties of the protein. Antibodies suitable for use in ELISA assays are commercially available from, for example, Amano Pharmaceutical Co. or may be raised against the IGF and/or binding protein of interest by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. Monoclonal antibodies may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol. 6*:511-519, 1976, and improvements thereto.

A secondary bioassay, based on the activity of the binding protein of interest, may also be employed for further characterization of a small molecule inhibitor or other ligand inhibitor. For example, IGF-I stimulates 3T3 fibroblast proliferation *in vitro*. The addition of a binding protein inhibits this stimulation, and the level of inhibition can be determined using [^{3}H]thymidine incorporation assays well known to those of ordinary skill in the art. Molecules that are capable of reversing the binding protein inhibition are ligand inhibitors. Similar assays may be designed for use with specific binding proteins based on the known biological properties of the binding proteins (*e.g.*, the inhibition of granulosa cell steroidogenesis as described in Bicsak et al., *Endocrinol. 126*:2184-2189, 1990).

Animal models may be useful for further characterization of ligand inhibitors. For example, the effect of a ligand inhibitor on blood glucose levels may be evaluated using animals, such as rats. An inhibitor that normalizes blood glucose levels in hyperglycemic or diabetic animals may be useful for the treatment of diabetes.

Similarly, growth hormone deficient animals may be used to evaluate the utility of a ligand inhibitor for the treatment of conditions that respond to the administration of human growth hormone, such as human growth hormone resistance.

Ligand inhibitors of the present invention may generally be used to increase the level of free, biologically active IGF in a patient and to treat any of a variety of IGF-responsive conditions. The term "IGF-responsive condition" encompasses any condition of a patient that may be alleviated or treated by the administration of IGF, and includes diseases such as diabetes (insulin dependent, non-insulin dependent and type I/II), growth retardation, osteoporosis, human growth hormone resistance, ALS, demyelinating diseases (including via remyelination), multiple sclerosis, muscular dystrophy, stroke, ophthalmic conditions, infertility, Alzheimer's disease and other dementias. The term "IGF-responsive condition" also encompasses states in which it is desirable to induce wound healing or bone repair, such as bone remodeling. As used herein, a "patient" refers to any warm-blooded animal, preferably a human. A patient may or may not be afflicted with an IGF-responsive condition. Patients that are so afflicted may generally be identified through clinical diagnosis according to methods that are well known to those of ordinary skill in the art.

Within the context of the present invention, the minimum acceptable increase in the level of free IGF is 10%, a preferred level is at least 50% and a particularly preferred level is at least 80%. The level of free IGF may generally be determined by methods known in the art, such as resolving the plasma sample to different molecular size fractions on a Sephadex G-50 fine column developed in 0.02M potassium phosphate buffer, pH 7.2. The free IGF-I with a molecular weight of 7.6 kDa will elute later than the larger molecular weight IGF-I/IGFBP complex. The free IGF-I fraction can then be quantitated by radioimmunoassay. Alternatively, blood glucose levels may be used as an indirect measurement of free IGF levels.

Other techniques, such as MRI, PETSCAN, spectacanning or other similar imaging techniques, may also be employed to measure the increase in the level of free IGF. Some of these techniques use radiolabeled ligand to IGF binding proteins or IGF receptors. A preferred method is image analysis using PET positron-emitting

ligands (*e.g.*, ^{11}C or ^{18}F) of a single photon-emitting ligand (*e.g.*, ^{123}I-labeled ligand to IGF-binding proteins or IGF receptors). Free IGF levels are correlated to the amount of binding of the radiolabeled ligand. An increase in IGF levels is manifested by a decreased binding of the radiolabeled ligand to the IGF-binding proteins and IGF receptors. Within this imaging technique, an increase in free IGF levels of about 10-30% or more is sufficient.

For administration to a patient, the ligand inhibitors are preferably incorporated into pharmaceutical compositions, which comprise a therapeutically effective amount of one or more ligand inhibitors and a physiologically acceptable carrier. A "physiologically acceptable carrier" may be any composition, carrier or diluent that is capable of administration to a mammal without producing undesirable physiological effects, such as nausea, dizziness or gastric upset. While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. In general, the pharmaceutical compositions may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax and/or a buffer. For oral administration, any of the above carriers and/or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed.

Small molecule inhibitors of the present invention are preferably capable of oral administration in, for example, capsule or tablet form. Such ligand inhibitors have the advantage of decreased cost and increased convenience, as compared to conventional treatments that rely on repeated intravenous injection. In general, the ability of a small molecule inhibitor to be administered orally may be determined by *in vivo* assays. Such assays typically measure the decrease in the level of IGF bound to binding proteins in response to oral administration of the small molecule inhibitor. For example, the amount of small molecule inhibitor in the blood may be measured based on its ability to inhibit binding of ^{125}I-hIGF-I to IGF binding proteins. Blood samples may be drawn at various times after administration of small molecule inhibitor or

vehicle to animals or humans. If necessary, the ligand inhibitor may be extracted from the blood by standard procedures (*e.g.*, 80% acetonitrile/0.1% trifluoroacetic acid). Briefly, 1 mL of the extraction solvent may be added to 200 μL of plasma and the mixture is vortexed. The resultant precipitate may then be centrifuged at 12,000 x g for 5 minutes. The supernatant containing the ligand inhibitor may then be lyophilized and reconstituted in assay buffer (PBS containing 0.2% Nonidet P-40™) to yield a reconstituted extract. Blood samples or reconstituted extracts may then be used in a binding assay, such as that described in Example 1, below. Ligand inhibitors capable of oral administration will show inhibition of binding of ^{125}I-hIGF-I to IGF binding proteins, relative to the vehicle control.

Routes and frequency of administration, as well as dosage, will vary depending on the ligand inhibitor and the desired *in vivo* response. A suitable dose is an amount of ligand inhibitor that, when administered as described above, is capable of improving the clinical outcome for a patient (*e.g.*, fewer hypoglycemic episodes for diabetic patients) in treated patients as compared to untreated patients.. In general, for pharmaceutical compositions comprising one or more ligand inhibitors, the amount of each ligand inhibitor present in a dose ranges from about 0.1 to about 10, preferably from about 1 to about 3 mg/kg. A larger or smaller amount may, however, be employed, depending on the size of the ligand inhibitor. Treatments are typically conducted one-two times per day, and may need to be continuous for retention of benefit. Patients may be monitored by assessing IGF levels as described above and by evaluating clinical symptoms.

A significant advantage of the present invention lies in the ability to vary the potency of the pharmaceutical composition and to target IGF effects to specific tissues, minimizing side effects. The potency may be varied through the use of ligand inhibitors with varying abilities to inhibit the binding of IGF to one or more binding proteins. In this regard, the relative abilities of ligand inhibitors to inhibit binding may be evaluated by, for example, comparing the amount of inhibitor needed for half maximal displacement of specific binding in an *in vitro* binding assay as described herein.

IGF effects may be targeted by using ligand inhibitors that are specific for one or more particular binding proteins, and exploiting the relative tissue specificity of each of the six known binding proteins (*see* Rechler, *Vitamins and Hormones* 47:1-114, 1993). Binding protein-specific ligand inhibitors may be developed, as noted above, by using different binding proteins within the binding assay described herein for the identification of small molecule inhibitors. Administration of such ligand inhibitors results in the release of IGF in only those tissues that contain the targeted binding proteins. For example, IGFBP-2, -4 and -6 are more prevalent in the brain. In addition, IGFBP-1 is present in the brain, although at lower concentrations. Small molecule inhibitors that are specific for these binding proteins and are capable of crossing the blood/brain barrier (as discussed below) will release IGF in the brain, while leaving most of the serum IGF in its inactive form. Such inhibitors may be particularly useful for the treatment of ALS and other neurodegenerative diseases such as multiple sclerosis, demyelinating disease and Alzheimer's disease. Alternatively, peripheral effects may be manipulated using primarily IGFBP-1, -3, -4, and -5. As noted above, circulating IGF-I may be released by inhibiting binding to IGFBP-3. In this regard, small molecule inhibitors that are not capable of crossing the blood/brain barrier are particularly well suited for providing peripheral effects.

The amount of a small molecule inhibitor that crosses the blood/brain barrier may be assessed by techniques known to those of ordinary skill in the art, such as MRI, PETSCAN, spectacanning or other similar imaging techniques, some of which use radiolabeled ligand to IGF binding proteins or IGF receptors. As noted above, a preferred method is image analysis using PET positron-emitting ligands (*e.g.*, ^{11}C or ^{18}F) of a single photon-emitting ligand (*e.g.*, ^{123}I-labeled ligand to IGF-binding proteins or IGF receptors). Decreased binding of the radiolabeled ligand to the IGF-binding proteins and IGF receptors indicates an increase in IGF levels. Such decreased binding is indicative of the level of small molecule inhibitor that has crossed the blood/brain barrier. Alternatively, IGF-I levels in the cerebrospinal fluid (CSF) can be measured by radioimmunoassay using commercially available assay kits (*e.g.*, Peninsula Laboratories, Inc., Belmont, CA) or by polyethylene/glycol precipitation, as described

in Example 1. An increase in the level of IGF-I in the CSF is indicative of an increase in the level of IGF-I in the brain.

In animals, the blood/brain penetration of the small molecule inhibitors can be tested by *ex vivo* binding. Briefly, animals may be injected (intravenously or orally) with 15-50 mg/kg of a small molecule ligand inhibitor. The animals are then sacrificed at 15, 30, 60 and 90 minutes after administration of the drug. The brains are removed and homogenized in solubilization buffer (PBS containing 0.2% Nonidet P-40™ detergent). The assay is then carried out by polyethyleneglycol precipitation of the bound ^{125}I-hIGF/hIGFBP-3 complex. If any drug is present in the brain, it will inhibit binding of the ^{125}I-hIGF-I to the IGF binding proteins, resulting in fewer precipitated counts in the drug-treated animals than in animals treated with vehicle alone.

Those of ordinary skill in the art will recognize that other tissues, and thus other IGF responsive conditions, may be targeted by administering ligand inhibitors specific for other binding proteins, or combinations thereof. The tissue distribution of the six known IGF binding proteins is presented in Table I, below:

Table I

Primary Tissue Distribution of IGF Binding Proteins

IGF Binding Protein	Tissues
BP-1	placenta, liver
BP-2	CSF, CNS, liver
BP-3	ovary, adrenal, heart, kidney, liver, stomach, intestine
BP-4	liver, brain cortex
BP-5	liver, brain, lung, heart, spleen, stomach, kidney, adrenal, intestine
BP-6	CSF and all tissue

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

Inhibition of IGF/BP-3 Binding Using hIGF-I Analogs

This Example illustrates the *in vitro* and *in vivo* inhibition of binding of IGF-I to BP-3, resulting in increased levels of free, biologically active IGF-I.

A. *In vitro* Binding Assay

The binding assay was performed in duplicate at room temperature in 0.2% BSA-PBS, pH 7.2. BP-3 was purified from rat serum as described in Shimonaka et al., *Biochem. Biophys. Res. Comm. 165*:189-195, 1989. Two hundred microliters of a 2.5 nM BP-3 solution (0.5 pmol) was added to a 12 x 75-mm glass tube. The reaction was started by the addition of an increasing concentration of unlabeled [T59]hIGF-I or [L24,59,60,A31]hIGF-I (both prepared by chemical synthesis according to the procedure described in Shimasaki et al., J. Biol. Chem. 266:10646-10653, 1991) in 100 µL followed by 100 µL of ^{125}I-labeled [T59]hIGF-I (30,000 cpm/~0.3 ng). After incubation for 2.5 hours, 100 µL of 20% BSA and 500 µL of 20% PEG-8000 in phosphate buffered saline (PBS) was added and the mixture was vortexed and then centrifuged for 30 minutes at 3500 rpm. The supernatant was carefully removed by suction and the pellet counted in a gamma counter.

As shown in Figure 1, 0.5 pmol of IGFBP-3 could bind 23.3% of the ^{125}I-labeled IGF-I tracer, and the non-specific binding was 1.8%. Half-maximal displacement of the specific binding was achieved at approximately 0.32 pmol [T59]hIGF-I per tube and 0.8 pmol [L24,59,60,A31]hIGF-I per tube (Figure 1).

This assay may also be employed to screen for small molecule ligand inhibitors.

B. [^{3}H]-Thymidine Incorporation Assay

BALB/c 3T3 cells were trypsanized and diluted to 50,000 cells per mL in 10% calf serum-DMEM. The cells were aliquoted to 96-well microtiter plates (180 µL/well). After 48 hours incubation at 37°C and 5% CO_2, the plates were washed twice with 0.1% calf serum-DMEM and incubated for an additional 24 hours. To each well, 20 µL of sample(s) containing IGF analog and/or binding protein and 1 µCi [^{3}H]-thymidine were added and the plates were incubated for precisely 24 hours. After the incubation, the medium was removed and the cells were fixed by adding 200 µL of 25% acetic acid-75% ethanol per well. After removal of the fixing solution the plates were washed three times with cold 10% TCA and the cells in each well were lysed in 200 µL 0.2M NaOH. The entire 200 µL of lysed solution was transferred into a scintillation vial and 2.5 mL scintillation liquid was added and the vials counted in a β-counter.

[T59]hIGF-I dose dependently stimulated proliferation, as measured by DNA synthesis, in 3T3 cells with a ED_{50} of 10-20 nM (Figure 2), whereas [L24,59,60,A31]hIGF-I did not induce any DNA synthesis in 3T3 cells with a dose as high as 8,000 nM (Figure 2). After incubation with 10 nM [T59]hIGF-I, the incorporated [^{3}H]-thymidine in the 3T3 cells was increased 4-fold in comparison with the control (Figure 3). Incubation with 25 nM IGFBP-3 completely inhibited the [^{3}H]-thymidine incorporation induced by 10 nM [T59]hIGF-I (Figure 3). Addition of [L24,59,60,A31]hIGF-I (which only binds to IGFBP-3, and not to the IGF-I receptors) could totally reverse the blocking effect of 25 nM IGFBP-3 to release the [T59]hIGF-I bioactivity with a ED_{50} of about 25 nM [L24,59,60,A31]hIGF-I (Figure 3).

This assay may also be employed to evaluate the biological activity of small molecule ligand inhibitors.

Example 2

Normalization of Blood Glucose Levels in Hyperglycemic Rats using IGFBP-3 Inhibitor

This Example illustrates the use of ligand inhibitors of IGFBP-3 for the treatment of animals with elevated blood glucose.

Rats were made hyperglycemic with an intraperitoneal injection of glucose. The [L24,59,60,A31]hIGF-I analog (noted as IGFBP3-LI) (5μg/min) was infused intravenously for 40 minutes and blood glucose levels were monitored before, during and after the infusion. As a control, bovine insulin was infused at 1 μg/min for 40 minutes and blood glucose was monitored at the same time points.

As depicted in Figure 5, while the insulin dramatically lowered blood glucose levels below the normal baseline, making the animals hypoglycemic, the [L24,59,60,A31]hIGF-I analog surprisingly normalized blood glucose levels. In a related experiment, insulin dramatically decreased blood glucose levels on a normal fasted blood glucose baseline, but [L24,59,60,A31]hIGF-I had no effect.

These results indicate that [L24,59,60,A31]hIGF-I can normalize blood glucose levels in animals with elevated blood glucose but, unlike insulin, this analog does not decrease the blood glucose levels below the normal baseline and make the animals hypoglycemic. Thus, the use of inhibitors of IGF binding to IGFBP-3 have distinct advantages over insulin for the treatment of diabetes. Diabetics receiving insulin normally experience a rebound effect after intravenous insulin injection such that blood sugar levels fall below normal levels. The inhibitors of the present invention normalize blood sugar levels without the risk of hypoglycemia. In addition, both insulin dependent and non-insulin dependent diabetics may be treated.

Example 3

Normalization of Blood Glucose Levels in Diabetic Mice using IGFBP-3 Inhibitor

This Example illustrates the use of ligand inhibitors of IGFBP-3 for the treatment of diabetes in animals.

Two severely diabetic female NOD mice with blood glucose levels of 450-500 mg/dl were treated with the [L24,59,60,A31]hIGF-I analog. These mice were severely diabetic and were expected to die within two days without treatment. The [L24,59,60,A31]hIGF-I analog was administered at time 0 (25 μg/animal), at 30 minutes (50 μg/animal) and at 60 min (100 μg/animal) by tail vein injection in physiological saline). Blood glucose levels were monitored using a glucometer before the initial injection and throughout the time course of the experiment.

In both animals, the blood glucose levels decreased dramatically to 250 mg/dl after 80 minutes (Figure 4). Since the threshold for normal blood glucose is 220 mg/dl, the blood glucose levels were almost normalized in both animals. These results demonstrate that the [L24,59,60,A31]hIGF-I analog may be used to lower blood glucose levels in the treatment of diabetes.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: DOMINIC P. BEHAN, NICHOLAS LING, XIN-JUN LIU AND AMITABH GAUR

(ii) TITLE OF INVENTION: LIGAND INHIBITORS OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND METHODS OF USE THEREFOR

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SEED and BERRY LLP
(B) STREET: 6300 Columbia Center, 701 Fifth Avenue
(C) CITY: Seattle
(D) STATE: Washington
(E) COUNTRY: USA
(F) ZIP: 98104-7092

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 17-APR-1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Maki, David J.
(B) REGISTRATION NUMBER: 31,392
(C) REFERENCE/DOCKET NUMBER: 690068.425

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (206) 622-4900
(B) TELEFAX: (206) 682-6031

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 70 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly
            20                  25                  30

Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys
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Lys Pro Ala Lys Ser Ala
65                  70
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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        35                  40                  45

Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Leu Leu Cys Ala Pro Leu
    50                  55                  60

Lys Pro Ala Lys Ser Ala
65                  70
```

Claims

1. A ligand inhibitor that inhibits the binding of an insulin-like growth factor to one or more insulin-like growth factor binding proteins, for use within a method for increasing the level of free, biologically active insulin-like growth factor in a patient.

2. The ligand inhibitor of claim 1 wherein the ligand inhibitor is [L24,59,60,A31]hIGF-I or a variant thereof that differs only in conservative substitutions and/or modifications.

3. The ligand inhibitor of claim 1 wherein the ligand inhibitor is a small molecule inhibitor.

4. The ligand inhibitor of claim 1 wherein the level of free, biologically active insulin-like growth factor increases in the patient's blood.

5. The ligand inhibitor of claim 1 wherein the level of free, biologically active insulin-like growth factor increases in the patient's brain.

6. The ligand inhibitor of claim 1 wherein the insulin-like growth factor is IGF-I.

7. The ligand inhibitor of claim 1 wherein the insulin-like growth factor is IGF-II.

8. The ligand inhibitor of claim 1 wherein the insulin-like growth factor binding protein is IGFBP-3.

9. The ligand inhibitor of claim 1 wherein the insulin-like growth factor binding protein is selected from the group consisting of IGFBP-1, IGFBP-2, IGFBP-4, IGFBP-5, IGFBP-6 and combinations thereof.

10. A ligand inhibitor that inhibits the binding of an insulin-like growth factor to one or more insulin-like growth factor binding proteins, for use within a method for treating an IGF-responsive condition in a patient.

11. The ligand inhibitor of claim 10 wherein the ligand inhibitor is [L24,59,60,A31]hIGF-I or a variant thereof that differs only in conservative substitutions and/or modifications.

12. The ligand inhibitor of claim 10 wherein the ligand inhibitor is a small molecule inhibitor.

13. The ligand inhibitor of claim 10 wherein the insulin-like growth factor is IGF-I.

14. The ligand inhibitor of claim 10 wherein the insulin-like growth factor is IGF-II.

15. The ligand inhibitor of claim 10 wherein the insulin-like growth factor binding protein is IGFBP-3.

16. The ligand inhibitor of claim 10 wherein the insulin-like growth factor binding protein is selected from the group consisting of IGFBP-1, IGFBP-2, IGFBP-4, IGFBP-5, IGFBP-6 and combinations thereof.

17. The ligand inhibitor of claim 10 wherein the IGF-responsive condition is selected from the group consisting of diabetes, growth retardation osteoporosis, human

growth hormone resistance, wounds, bone damage, ALS, Alzheimer's disease, demyelinating disease, multiple sclerosis, muscular dystrophy, stroke and neuronal degeneration.

18. A pharmaceutical composition, comprising:

(a) one or more ligand inhibitors that inhibit the binding of an insulin-like growth factor to one or more insulin-like growth factor binding proteins; and

(b) a physiologically acceptable carrier.

19. A pharmaceutical composition according to claim 18 wherein the ligand inhibitor is [L24,59,60,A31]hIGF-I or a variant thereof that differs only in conservative substitutions and/or modifications.

20. A pharmaceutical composition according to claim 18 wherein the ligand inhibitor is a small molecule inhibitor.

21. A method for screening for a small molecule inhibitor that inhibits binding of an insulin-like growth factor to an insulin-like growth factor binding protein, comprising:

(a) combining an insulin-like growth factor with an insulin-like growth factor binding protein in a solution containing a candidate small molecule, such that the binding protein and the growth factor are capable of forming a complex; and

(b) determining the amount of complex in the solution, relative to a predetermined level of binding in the absence of the small molecule, and therefrom evaluating the ability of the small molecule to inhibit binding of an insulin-like growth factor to an insulin-like growth factor binding protein.

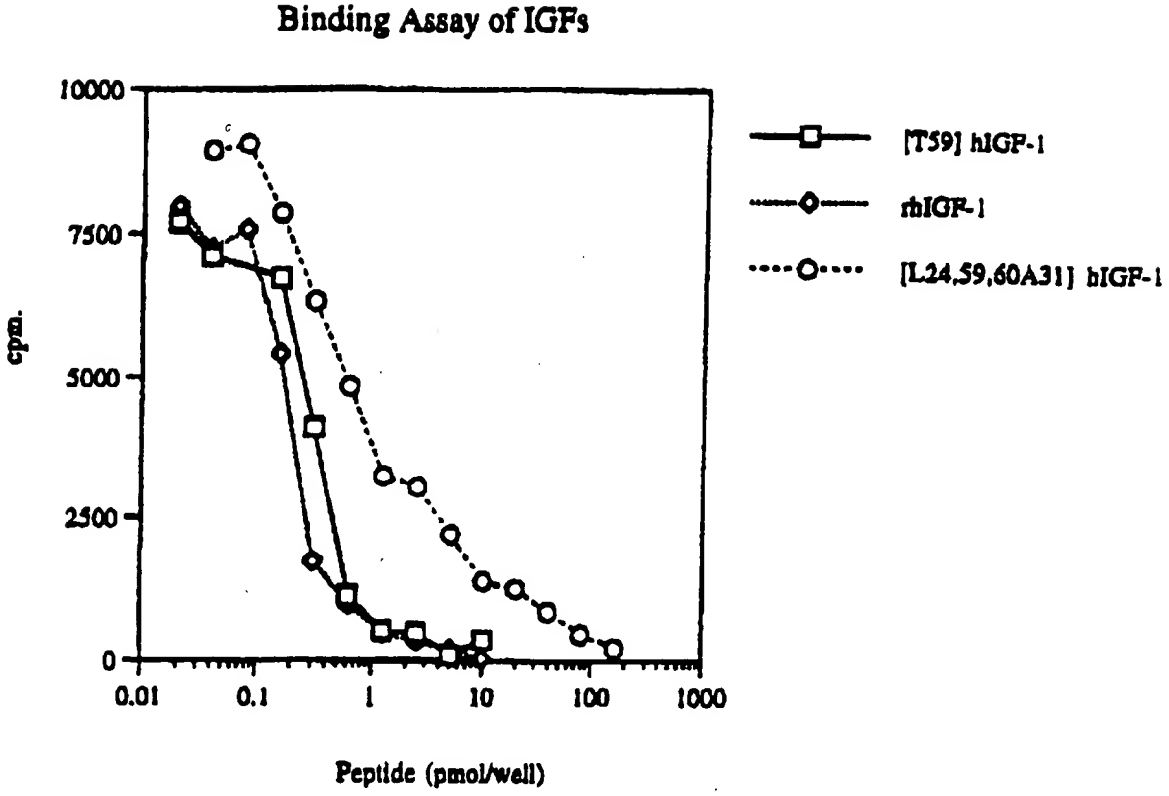
22. The method of claim 21 wherein the insulin-like growth factor is IGF-I.

23. The method of claim 21 wherein the insulin-like growth factor binding protein is IGFBP-3.

24. The method of claim 21 wherein the insulin-like growth factor binding protein is selected from the group consisting of IGFBP-1, IGFBP-2, IGFBP-4, IGFBP-5, IGFBP-6 and combinations thereof.

25. A ligand inhibitor that inhibits the binding of the protein to one or more insulin-like growth factor binding proteins, for use within a method for increasing the level of a free, biologically active protein in a patient.

26. A ligand inhibitor that inhibits the binding of insulin-like growth factor to one or more insulin-like growth factor binding proteins, for use within a method for increasing the level of free IGF-II in a patient.


Binding Assay of IGFs
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7500
5000
2500
0
cpm.
0.01
0.1
1
10
100
1000
Peptide (pmol/well)
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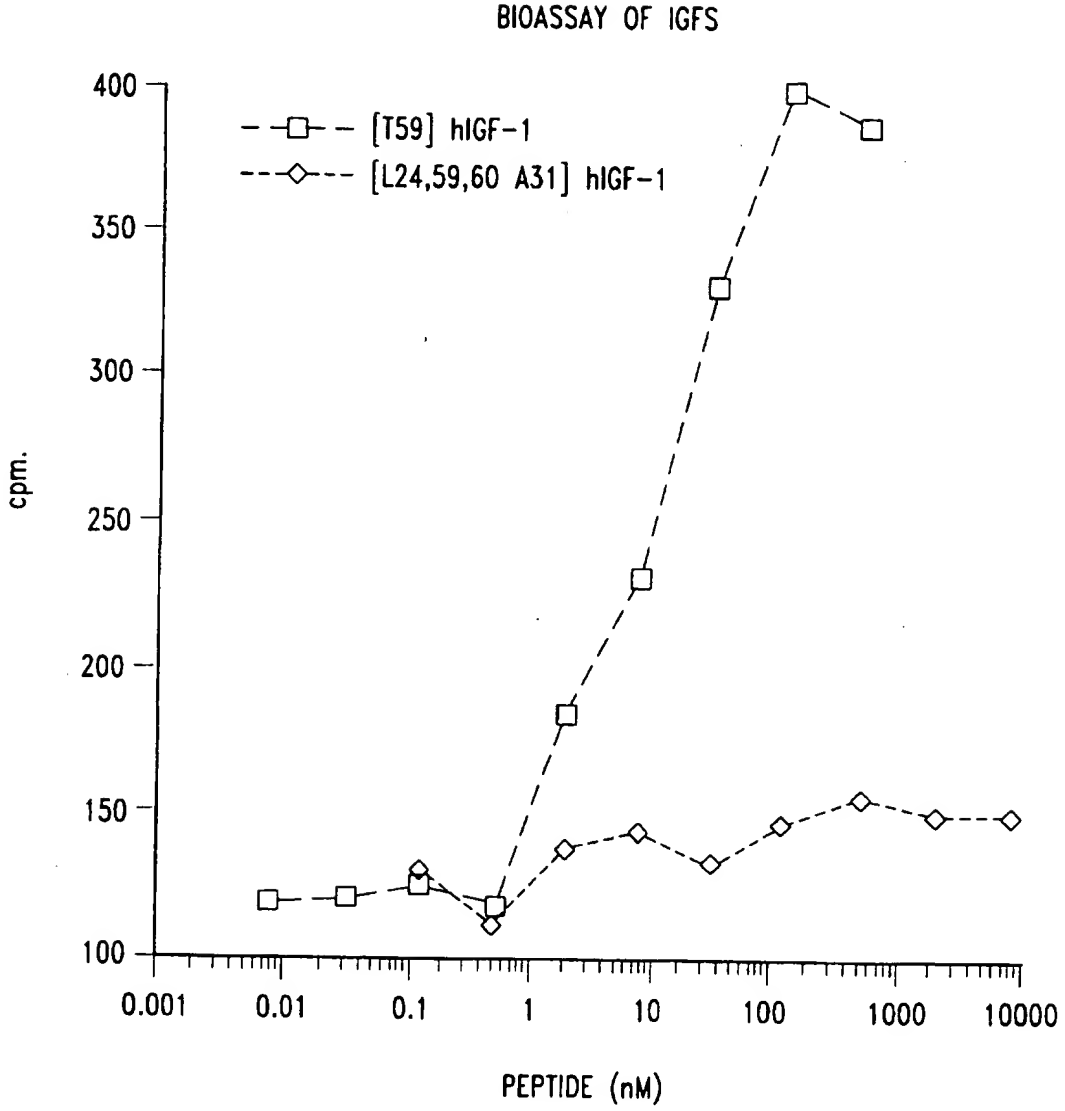
rhIGF-1
[L24,59,60A31] hIGF-1

Fig. 1


BIOASSAY OF IGFS
[T59] hIGF-1
[L24,59,60 A31] hIGF-1
cpm.
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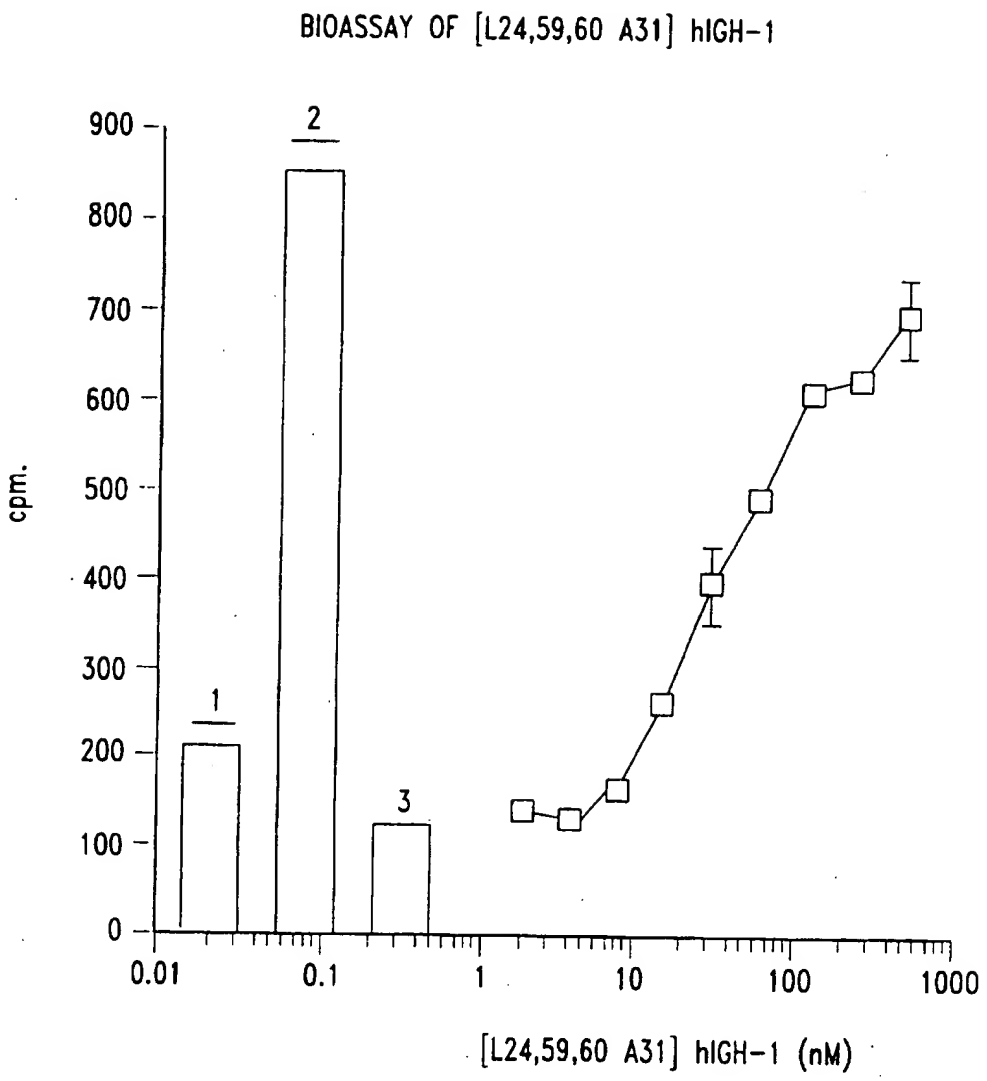
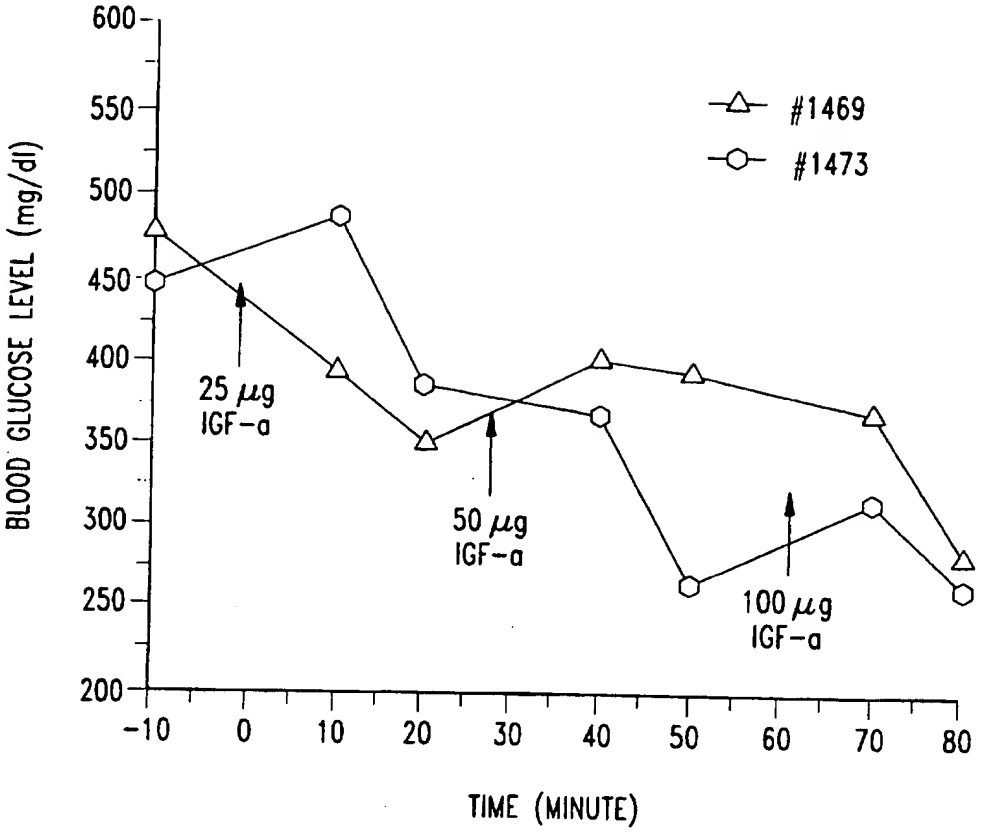
350
300
250
200
150
100
0.001
0.01
0.1
1
10
100
1000
10000
PEPTIDE (nM)

Fig. 2



COLUMN 1: MEDIUM ONLY
COLUMN 2: 10 nM [T59] hIGF-1
COLUMN 3: 10nM IGF-1+25 nM BP-3

Fig. 3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550
500
450
400
350
300
250
200
BLOOD GLUCOSE LEVEL (mg/dl)
#1469
#1473
25 μg IGF-a
50 μg IGF-a
100 μg IGF-a
-10
0
10
20
30
40
50
60
70
80
TIME (MINUTE)

Fig. 4

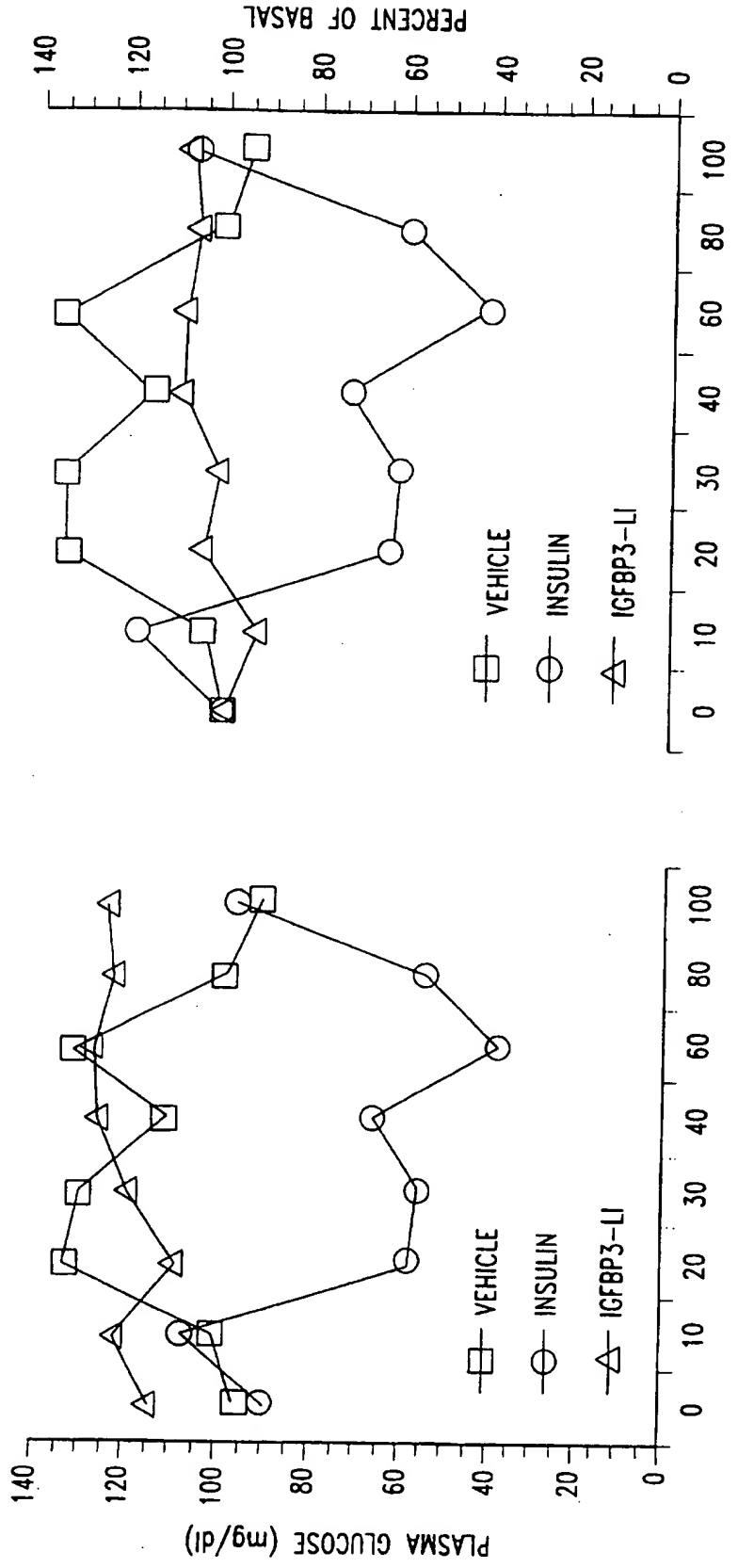
PERCENT OF BASAL
140
120
100
80
60
40
20
0
0
10
20
30
40
60
80
100
TIME (MIN.)
VEHICLE
INSULIN
IGFBP3-LI
PLASMA GLUCOSE (mg/dl)

Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/06503

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/65 A61K38/30 G01N33/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 26, 15 September 1990, MD US, pages 15648-15652, XP002039492 BAYNE E.A.: "The role of tyrosines 24,31 and 60 in the high affinity binding of IGF-1 to the type 1 IGF-R"	1-26
Y	see the whole document ---	2,11,19
X	EP 0 135 094 A (AMGEN) 27 March 1985	1,3-10, 12-18, 20-26
Y	The whole document; see esp. p.14, lines 17-20; claim 38 see the whole document ---	2,11,19
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
2 September 1997	19.09.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Groenendijk, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMISTRY, vol. 3, no. 9, 3 May 1988, EASTON, PA US, pages 3229-3233, XP002039493 CASCIERI E.A.: "Mutants of hIGF-1 with reduced affinity for the type 1 IGF-R" see the whole document ---	1,3-10, 12-18, 20-26
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, no. 4, 5 February 1989, pages 2199-2202, XP000009847 CASCIERI M A ET AL: "STRUCTURAL ANALOGS OF HUMAN INSULIN-LIKE GROWTH FACTOR (IGF) I WITH ALTERED AFFINITY FOR TYPE 2 IGF RECEPTORS" see the whole document ---	1,3-10, 12-18, 20-26
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, no. 32, 15 November 1989, pages 19155-19160, XP000081891 HAMPTON B ET AL: "PURIFICATION AND CHARACTERIZATION OF AN INSULIN-LIKE GROWTH FACTOR II VARIANT FROM HUMAN PLASMA" see the whole document -----	1,3-10, 12-18, 20-26

INTERNATIONAL SEARCH REPORT

ernat l application No.
PCT/US 97/06503

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Please see Further Information sheet enclosed.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Obscurities :

The scope of the claims 1,3-10,12-18 and 20-26 is unclear and speculative. Said claims lack any indication concerning the structural requirements of the "ligand inhibitors".

Furthermore the available experimental data actually only relate to a structural analog of IGF. Therefore the claims can also not be considered to represent a permissible generalisation which is fairly based on experimental evidence, that is, they are also not adequately supported by the description (Art.6 PCT).

Therefore a meaningful and economically feasible search could not encompass the complete subject-matter of the claims. Consequently the search has been directed to compounds structurally related to IGF or IGF-BP and has only been complete for the subject-matter of the claims 2,11 and 19 (Art.17(2)(a)(ii) PCT).

Incomplete Search

Claims searched completely : 2, 11, 19

Claims searched incompletely : 1, 3-10, 12-18, 20-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/06503

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0135094 A	27-03-85	EP 0406913 A	09-01-91
		JP 60501989 T	21-11-85
		WO 8500831 A	28-02-85